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(54) Title: T2K KINASE ASSAYS

(57) Abstract: T2K kinase activity is detected by forming a mixture of a T2K kinase and a substrate; incubating the mixture under conditions whereby the kinase phosphorylates the substrate at a first rate; and detecting the first rate as an indication of the kinase activity. The substrate comprises SX₁X₂X₃SX₄ (SEQ ID NO:1) wherein X₁ and X₄ are aliphatic residues and both of the S residues are targets of the kinase, and especially, IKK α or IKK β . In another embodiment, the mixture substrate comprises a particular IL-1 or TNF signaling cascade component. The mixture may be used to screen for agents which modulate the activity of the kinase, e.g. as an immuno-chemiluminescent assay.

T2K Kinase Assays

Inventor: Zhaodan Cao, of South San Francisco, CA

INTRODUCTION

Field of the Invention

The field of this invention is detecting the activity of an enzyme.

Background

Inflammatory cytokines IL-1 and TNF exert diverse biological activities by altering gene expression in the cells, a function mediated in part by transcription factor NF- κ B. In unstimulated cells, NF- κ B proteins form a complex with inhibitory molecules, the I κ B proteins, and are rendered inactive in the cytoplasm. In response to cytokines and other stimuli, the I κ B proteins are phosphorylated on specific serine residues. Delineating TNF and IL-1 signaling pathways for NF- κ B activation has implicated the TRAF molecules as converging point for different cytokines, with TRAF2 being involved in TNF- and TRAF6 in IL-1-induced NF- κ B activation. We previously disclosed a family of I κ B kinases including a TRAF2-associated kinase activity (designated T2K) and the translation product of the KIAA0151 gene product (also known as IKKi and IKK ϵ) that phosphorylates the I κ B molecules on the specific regulatory serine residues. We have now found that T2K and IKKi in fact have alternative substrate specificities and alternative physiologically relevant substrate targets, particularly IKK α and IKK β peptide substrates. We disclose here materials and methods for assaying for these novel specificities.

Relevant Literature

T2K (also known as TBK1) is described by Cao et al. (US Patent No.5,776,717) and Pomerantz and Baltimore, EMBO J. 18 (23), 6694-6704, 1999; Genbank Accession No. NM_013254 and NP_037386 (human); AF191839 and AAF05990.1 (mouse).

IKKi is described by Nagase *et al.*, DNA Res., 1995, 2, 167-174; Genbank Accession Nos. D63485 and BAA09772 (human); and by Shimada et al., Int Immunol. 1999 Aug;11(8):1357-62; Genbank Accession Nos. AB016590 and BAA85155.1 (human); AB016589 and BAA85154.1 (mouse).

IKK α is described in Regnier et al. 1997, Cell 90, 373-383; Genbank Accession Nos.AF012890 and AAC51662.1 (human).

IKK β is described in US Patent No. 5,939,302; Genbank Accession Nos.AF029684 and AAC51860.1 (human).

Song et al., US Patent No.5,874,230 disclose a TRAF2-associated kinase.

SUMMARY OF THE INVENTION

The invention provides compositions and methods for detecting kinase activity. The subject compositions include in vitro mixtures comprising or consisting essentially of an isolated, active T2K kinase and an isolated, functional T2K substrate. In one embodiment, the substrate comprises or consists essentially of SX₁X₂X₃SX₄ (SEQ ID NO:1) wherein X₁ and X₄ are aliphatic residues and both of the S residues are targets of the kinase. In particular aspects, X₁ and X₄ are L and F, respectively; X₁-X₄ are L, C, T and F, respectively; the substrate comprises a sequence selected from the group consisting of YAKDVDQGSLCTSFGVTLQYL (SEQ ID NO:2) and YAKELDQGSLCTSFGVTLQYL (SEQ ID NO:3); and/or the substrate comprises a natural human kinase selected from the group consisting of IKK α and IKK β . In another embodiment, the substrate comprises or consists essentially of an IL-1 or TNF signaling cascade component selected from the group consisting of: an isolated natural human IL1 Rc superfamily receptor selected from the group consisting of IL-1RP1, IL-1RP2, IL-1RP3, IL-18Rc, TLR2 and TLR4; a natural human NfkB protein selected from the group consisting of p50, p65, p49, cRel and RelB; a natural human protein selected from the group consisting of I-TRAF and IKK γ ; a natural human protein selected from the group consisting of TRAF5 and TRAF6; a natural human protein selected from the group consisting of RIP, IRAK, MYD88 and TRADD; and a natural human TNFRc1 protein selected from the group consisting of CD40 and CD30.

The subject methods for detecting kinase activity comprise the steps of forming a subject mixture, incubating the mixture under conditions whereby the kinase phosphorylates the substrate at a first rate, and detecting the first rate as an indication of the kinase activity. In particular aspects, the mixture comprises an agent and but for the presence of the agent, the kinase phosphorylates the substrate at a second rate, wherein a significant difference between the first and second rate is an indication that the agent modulates the kinase activity. In more

particular aspects, the detecting step is a chemiluminescent assay comprising detecting the phosphorylated substrate with a specific, labeled probe, particularly wherein the phosphorylated substrate is immobilized and detecting the phosphorylated substrate is effected indirectly by detecting a substrate-specific primary antibody with the probe, wherein the probe is a labeled secondary antibody specific for the primary antibody.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides a method for detecting kinase activity comprising the steps of (a) forming a mixture comprising an active T2K kinase and a T2K substrate; (b) incubating the mixture under conditions whereby the kinase phosphorylates the substrate at a first rate; and (c) detecting the first rate as an indication of the kinase activity. As used herein, the term T2K kinase describes the two related natural proteins T2K and natural IKKi, however insubstantial variants such as minor truncations, etc. which retain substantially the same kinase activity and specificity of the native proteins are clearly equivalents in the disclosed assays. The T2K kinases may be produced recombinantly and/or isolated from any convenient source, particularly human or murine cells, see e.g. Molecular Cloning, A Laboratory Manual (Sambrook, *et al.* Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, *et al.*, Greene Publ. Assoc., Wiley-Interscience, NY).

The T2K substrate is of sufficient length and sequence to provide a functional substrate for the T2K kinase under assay conditions. Depending on the assay structure, the substrate is generally a peptide of at least 5, preferably at least 10, more preferably at least 15 residues in length. In many cases, cost and specificity are optimized by using peptides of fewer than 100 residues, preferably fewer than 50 residues, more preferably fewer than 25 residues, as opposed to native protein substrates.

In one embodiment, the T2K substrate comprises SX₁X₂X₃SX₄ (SEQ ID NO:1), wherein X₁ and X₄ are aliphatic residues and both of the S residues are targets of the kinase, particularly wherein X₁ and X₄ are L and F, more particularly wherein X₁-X₄ are L, C, T and F, respectively. Table 1 provides exemplary peptides providing requisite T2K substrate specificity.

Table 1. Exemplary peptides providing requisite T2K substrate specificity.

Peptide	Sequence Identifier	T2K substrate specificity
GGSLCTSF	(SEQ ID NO:4)	+++
GGSLMTSF	(SEQ ID NO:5)	+++
GGSFCTSL	(SEQ ID NO:6)	+++
DQGSFCTSL	(SEQ ID NO:7)	+++
VDQGSLAASFVGT	(SEQ ID NO:8)	+++
VDQGSMAASLVGT	(SEQ ID NO:9)	+++
VDQGSIAASFVGT	(SEQ ID NO:10)	+++
AKDVDQGSVCTSFGTLQY	(SEQ ID NO:11)	+++
AKDVDQGSLCTSFGTLQY	(SEQ ID NO:12)	+++
AKDVDQGSFCTSFGTLQY	(SEQ ID NO:13)	+++

In more particular embodiments the substrate comprises an IKK α fragment including serines 176 and 180 or an IKK β fragment including serines 177 and 181; hence, there are ten IKK α fragments and ten IKK β fragments 15 residues in length (IKK β resides 167-181, 168-182, etc.). Particular substrates include IKK α fragment YAKDVDQGSLCTSFGTLQYL (SEQ ID NO:2) and IKK β fragment YAKELDQGSLCTSFGTLQYL (SEQ ID NO:3).

Alternatively, native IKK α and IKK β substrates may be used.

In another embodiment, the T2K substrate comprises an IL-1 or TNF signaling cascade component other than I κ B and TRAF2, such as a natural human IL-1 R c superfamily receptor selected from the group consisting of IL-1RP1, IL-1RP2, IL-1RP3, IL-18R c , TLR2 and TLR4; a natural human Nf κ B protein selected from the group consisting of p50, p65, p49, cRel and RelB; a natural human protein selected from the group consisting of I-TRAF and IKK γ (also known as NEMO); a natural human protein selected from the group consisting of TRAF5 and TRAF6; a natural human protein selected from the group consisting of RIP, IRAK, MYD88 and TRADD; and a natural human TNFR c 1 protein selected from the group consisting of CD40 and CD30. These enumerated components are all defined in the art and readily isolated or produced recombinantly using sequence available through public repositories such as Genbank. In addition, insubstantial variants such as minor truncations, etc. which retain substantially the same kinase activity and specificity of the native proteins

are clearly equivalents in the disclosed assays.

After forming the mixture, the method involves incubating the mixture under conditions whereby the kinase phosphorylates the substrate at a first rate; and detecting the first rate as an indication of the kinase activity. Incubation conditions and periods are for kinase activity but also minimized to facilitate rapid, high-throughput screening. For continuous assays, the rate may be expressed in terms of a dynamic activity, whereas in most applications, the assays are discontinuous (e.g. have a fixed-time endpoint) so the rate is expressed as net kinase activity over a fixed time.

In a particular embodiment, the method is used to screen for agents which modulate the activity of the kinase, wherein the mixture comprises an agent and but for the presence of the agent, the kinase phosphorylates the substrate at a second rate, wherein a significant difference between the first and second rate is an indication that the agent modulates the kinase activity. A wide variety of homogeneous and heterogeneous (solid phase) assays may be used, including fluorescent polarization assays, homogeneous time resolved fluorescence assays (HTRF), capture assays such as described by Strulovici in US Pat No. 5,759,787, etc. In particular embodiments, the detecting step is a chemiluminescent assay comprising detecting the phosphorylated substrate with a specific, labeled probe, particularly wherein the phosphorylated substrate is immobilized and detecting the phosphorylated substrate is effected indirectly by detecting a substrate-specific primary antibody with the probe, wherein the probe is a labeled secondary antibody specific for the primary antibody.

The following experimental section and examples are offered by way of illustration and not by way of limitation.

EXAMPLES

1. Chemiluminescent Protein Kinase Assay.

A. MATERIALS:

- 1) Recombinant human T2K purified from baculovirus infected insect cells
- 2) Substrate: biotinylated peptide (SEQ ID NO:2)
- 3) Streptavidin coated microtiter plates: coated in-house
- 4) Primary antibody: anti-phosphoserine mAb, specific for epitope
- 5) Secondary antibody: HRP conjugated goat anti-mouse antibody

6) SuperSignal detection reagent

B. METHODS:

Plate coating:

1) Reconstitute streptavidin directly from powder in PBS at around 40 µg/ml and coat Dynatech Microlite-2 plates (120 µl/well) either O/N at 4°C or 2hr RT. The plate can be stored at 4°C for at least 3 days with no loss of binding capacity. Directly before use wash the plate 3x with 200 µl d.w.

Kinase reaction:

2) Reaction mixture contains in a final volume of 90 µl/well: 20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 2 mM EDTA, 1 mM NaF, 0.5 mM benzamidine, 1 mM DTT, 0.1% NP-40, 100 µM ATP, 1x10⁻⁶ M substrate.

3) Add test compound: 10 µl/well in DMSO.

4) Add enzyme in 10 µl buffer containing Tris-HCl pH 7.5 20 mM, 10 % DMSO, EDTA 2 mM, benzamidine 2 mM, DTT 1 mM, NP-40 0.1%, MgCl₂ 1 mM.

5) Incubate at room temperature for 40 min.

ELISA reaction:

6) Wash 3x with 300 µl PBS.

7) Add 100 µl/well primary antibody (1:10,000) premixed with secondary antibody (1: 5,000) in PBS containing 2% BSA. Incubate at room temperature for 40 min on shaker.

8) Wash 3x with 300 µl PBS.

Detection:

9) Add 100 µl/well 3x diluted SuperSignal substrate. Incubate at RT for 10 min and read by luminometer.

2. Continuous High-Throughput In Vitro Fluorescence Polarization Assay.

Sensor: Rhodamine-labeled peptide (SEQ ID NO:3, final conc. = 1 - 5 nM)

Kinase: T2K (final conc. = 100 - 200 nM)

Buffer: 10 mM HEPES, 10 mM NaCl, 6 mM magnesium chloride, pH 7.6

1. Add 90 microliters of Sensor/Kinase mixture to each well of a 96-well microtiter plate.
2. Add 10 microliters of test compound per well.

3. Shake 5 min and within 5 minutes determine amount of fluorescence polarization by using a Fluorolite FPM-2 Fluorescence Polarization Microtiter System (Dynatech Laboratories, Inc).

3. Solid Phase IKKi Phosphorylation Assay

A. Reagents:

- Neutralite Avidin: 20 µg/ml in PBS.
- kinase: 10^{-8} - 10^{-5} M IKKi at 20 µg/ml in PBS.
- substrate: 10^{-7} - 10^{-4} M biotinylated substrate (SEQ ID NO:13) at 40 µg/ml in PBS.
- Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hour at room temperature.
- Assay Buffer: 100 mM KCl, 10 mM MgCl₂, 1 mM MnCl₂, 20 mM HEPES pH 7.4, 0.25 mM EDTA, 1% glycerol, 0.5% NP-40, 50 mM BME, 1 mg/ml BSA, cocktail of protease inhibitors.
- [³²P]γ-ATP 10x stock: 2 x 10^{-5} M cold ATP with 100 µCi [³²P]γ-ATP. Place in the 4°C microfridge during screening.

- Protease inhibitor cocktail (1000X): 10 mg Trypsin Inhibitor (BMB # 109894), 10 mg Aprotinin (BMB # 236624), 25 mg Benzamidine (Sigma # B-6506), 25 mg Leupeptin (BMB # 1017128), 10 mg APMSF (BMB # 917575), and 2mM NaVO₃ (Sigma # S-6508) in 10 ml of PBS.

B. Preparation of assay plates:

- Coat with 120 µl of stock N Avidin per well overnight at 4°C.
- Wash 2 times with 200 µl PBS.
- Block with 150 µl of blocking buffer.
- Wash 2 times with 200 µl PBS.

C. Assay:

- Add 40 µl assay buffer/well.
- Add 40 µl biotinylated substrate (2-200 pmoles/40 ul in assay buffer)
- Add 40 µl kinase (0.1-10 pmoles/40 ul in assay buffer)
- Add 10 µl compound or extract.
- Add 10 µl [³²P]γ-ATP 10x stock.
- Shake at 25°C for 15 minutes.

- Incubate additional 45 minutes at 25°C.
- Stop the reaction by washing 4 times with 200 µl PBS.
- Add 150 µl scintillation cocktail.
- Count in Topcount.

D. Controls for all assays (located on each plate):

- a. Non-specific binding
- b. cold ATP at 80% inhibition.

4. Solid Phase T2K Phosphorylation Assay.

A. Reagents:

- Neutralite Avidin: 20 µg/ml in PBS.
- kinase: 10⁻⁸ - 10⁻⁵ M T2K (residues 1-728) at 20 µg/ml in PBS.
- substrate: 10⁻⁷ - 10⁻⁴ M biotinylated substrate (SEQ ID NO:3) at 40 µg/ml in PBS.
- Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hour at room temperature.
- Assay Buffer: 100 mM KCl, 10 mM MgCl₂, 1 mM MnCl₂, 20 mM HEPES pH 7.4, 0.25 mM EDTA, 1% glycerol, 0.5% NP-40, 50 mM BME, 1 mg/ml BSA, cocktail of protease inhibitors.
- [³²P]γ-ATP 10x stock: 2 x 10⁻⁵ M cold ATP with 100 µCi [³²P]γ-ATP. Place in the 4°C microfridge during screening.

- Protease inhibitor cocktail (1000X): 10 mg Trypsin Inhibitor (BMB # 109894), 10 mg Aprotinin (BMB # 236624), 25 mg Benzamidine (Sigma # B-6506), 25 mg Leupeptin (BMB # 1017128), 10 mg APMSF (BMB # 917575), and 2mM NaVo₃ (Sigma # S-6508) in 10 ml of PBS.

B. Preparation of assay plates:

- Coat with 120 µl of stock N Avidin per well overnight at 4°C.
- Wash 2 times with 200 µl PBS.
- Block with 150 µl of blocking buffer.
- Wash 2 times with 200 µl PBS.

C. Assay:

- Add 40 µl assay buffer/well.
- Add 40 µl biotinylated substrate (2-200 pmoles/40 µl in assay buffer)

- Add 40 μ l kinase (0.1-10 pmoles/40 μ l in assay buffer)
- Add 10 μ l compound or extract.
- Add 10 μ l [32 P] γ -ATP 10x stock.
- Shake at 25°C for 15 minutes.
- Incubate additional 45 minutes at 25°C.
- Stop the reaction by washing 4 times with 200 μ l PBS.
- Add 150 μ l scintillation cocktail.
- Count in Topcount.

- D. Controls for all assays (located on each plate):
- a. Non-specific binding
 - b. cold ATP at 80% inhibition.

3. Protocol for high throughput T2K-TRAF2 heterodimer formation assay.

A. Reagents:

- Neutralite Avidin: 20 μ g/ml in PBS.
- Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hour at room temperature..
- Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.6, 1 mM MgCl₂, 1% glycerol, 0.5% NP-40, 50 mM β -mercaptoethanol, 1 mg/ml BSA, cocktail of protease inhibitors.
- 33 P T2K protein 10x stock: 10⁻⁸ - 10⁻⁶ M "cold" T2K supplemented with 200,000-250,000 cpm of labeled T2K (Beckman counter). Place in the 4°C microfridge during screening.
- Protease inhibitor cocktail (1000X): 10 mg Trypsin Inhibitor (BMB # 109894), 10 mg Aprotinin (BMB # 236624), 25 mg Benzamidine (Sigma # B-6506), 25 mg Leupeptin (BMB # 1017128), 10 mg APMSF (BMB # 917575), and 2mM NaVo₃ (Sigma # S-6508) in 10 ml of PBS.

- TRAF2: 10⁻⁷ - 10⁻⁵ M biotinylated TRAF2 in PBS.

B. Preparation of assay plates:

- Coat with 120 μ l of stock N-Avidin per well overnight at 4°C.
- Wash 2 times with 200 μ l PBS.
- Block with 150 μ l of blocking buffer.
- Wash 2 times with 200 μ l PBS.

C. Assay:

- Add 40 µl assay buffer/well.
- Add 10 µl compound or extract.
- Add 10 µl ^{33}P -T2K (20-25,000 cpm/0.1-10 pmoles/well = 10^{-9} - 10^{-7} M final conc).
- Shake at 25°C for 15 minutes.
- Incubate additional 45 minutes at 25°C.
- Add 40 µl biotinylated TRAF2 (0.1-10 pmoles/40 ul in assay buffer)
- Incubate 1 hour at room temperature.
- Stop the reaction by washing 4 times with 200 µM PBS.
- Add 150 µM scintillation cocktail.
- Count in Topcount.

D. Controls for all assays (located on each plate):

- a. Non-specific binding
- b. Soluble (non-biotinylated TRAF2) at 80% inhibition.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for detecting kinase activity comprising the steps of:
 - (a) forming a mixture comprising an active T2K kinase and a T2K substrate comprising SX₁X₂X₃SX₄ (SEQ ID NO:1), wherein X₁ and X₄ are aliphatic residues and both of the S residues are targets of the kinase;
 - (b) incubating the mixture under conditions whereby the kinase phosphorylates the substrate at a first rate; and
 - (c) detecting the first rate as an indication of the kinase activity.
2. A method according to claim 1, wherein X₁ and X₄ are aliphatic residues and both of the S residues are targets of the kinase;
3. A method according to claim 1, wherein X₁ and X₄ are L and F.
4. A method according to claim 1, wherein X₁-X₄ are L, C, T and F, respectively.
5. A method according to claim 1, wherein the substrate comprises a sequence selected from the group consisting of YAKDVDQGSLCTSFVGTLQYL (SEQ ID NO:2) and YAKELDQGSLCTSFVGTLQYL (SEQ ID NO:3).
6. A method according to claim 1, wherein the substrate comprises a natural human kinase selected from the group consisting of IKK α and IKK β .
7. A method according to claim 1, wherein the mixture comprises an agent and but for the presence of the agent, the kinase phosphorylates the substrate at a second rate, wherein a significant difference between the first and second rate is an indication that the agent modulates the kinase activity.
8. A method according to claim 1, wherein the detecting step is a chemiluminescent assay comprising detecting the phosphorylated substrate with a specific, labeled probe.

9. A method according to claim 1, wherein the detecting step is a chemiluminescent assay comprising detecting the phosphorylated substrate with a specific, labeled probe, wherein the phosphorylated substrate is immobilized and detecting the phosphorylated substrate is effected indirectly by detecting a substrate-specific primary antibody with the probe, wherein the probe is a labeled secondary antibody specific for the primary antibody.
10. A method according to claim 1, wherein the mixture comprises an agent and but for the presence of the agent, the kinase phosphorylates the substrate at a second rate, wherein a significant difference between the first and second rate is an indication that the agent modulates the kinase activity.
11. A method according to claim 1, wherein the detecting step is a chemiluminescent assay comprising detecting the phosphorylated substrate with a specific, labeled probe.
12. A method according to claim 1, wherein the detecting step is a chemiluminescent assay comprising detecting the phosphorylated substrate with a specific, labeled probe, wherein the phosphorylated substrate is immobilized and detecting the phosphorylated substrate is effected indirectly by detecting a substrate-specific primary antibody with the probe, wherein the probe is a labeled secondary antibody specific for the primary antibody.
13. A method according to claim 4, wherein the detecting step is a chemiluminescent assay comprising detecting the phosphorylated substrate with a specific, labeled probe, wherein the phosphorylated substrate is immobilized and detecting the phosphorylated substrate is effected indirectly by detecting a substrate-specific primary antibody with the probe, wherein the probe is a labeled secondary antibody specific for the primary antibody.
14. A method for detecting kinase activity comprising the steps of:
 - (a) forming a mixture comprising an active T2K kinase and a T2K substrate comprising an IL-1 or TNF signaling cascade component selected from the group consisting of:

a natural human IL-1 Rc superfamily receptor selected from the group consisting of IL-1RP1, IL-1RP2, IL-1RP3, IL-18Rc, TLR2 and TLR4;

a natural human Nf κ B protein selected from the group consisting of p50, p65, p49, cRel and RelB;

a natural human protein selected from the group consisting of I-TRAF and IKK γ ;

a natural human protein selected from the group consisting of TRAF5 and TRAF6;

a natural human protein selected from the group consisting of RIP, IRAK, MYD88 and TRADD; and

a natural human TNFRc1 protein selected from the group consisting of CD40 and CD30;

(b) incubating the mixture under conditions whereby the kinase phosphorylates the substrate at a first rate; and

(c) detecting the first rate as an indication of the kinase activity.

15. A method according to claim 14, wherein the mixture comprises an agent and but for the presence of the agent, the kinase phosphorylates the substrate at a second rate, wherein a significant difference between the first and second rate is an indication that the agent modulates the kinase activity.

16. A method according to claim 14, wherein the detecting step is a chemiluminescent assay comprising detecting the phosphorylated substrate with a specific, labeled probe.

17. A method according to claim 14, wherein the detecting step is a chemiluminescent assay comprising detecting the phosphorylated substrate with a specific, labeled probe, wherein the phosphorylated substrate is immobilized and detecting the phosphorylated substrate is effected indirectly by detecting a substrate-specific primary antibody with the probe, wherein the probe is a labeled secondary antibody specific for the primary antibody.

SEQUENCE LISTING

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